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Immune Selection for Antigenic Drift of Major Outer Membrane Protein P2 of *Haemophilus influenzae* during Persistence in Subcutaneous Tissue Cages in Rabbits

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During persistence of nonencapsulated *Haemophilus influenzae* in the respiratory tracts of patients with chronic bronchitis, the major outer membrane proteins (MOMPs) P2 and P5 show antigenic drift. The hypothesis that appearance of antigenic variants is the consequence of antibody-dependent selection was tested in a rabbit model. Persistence of *H. influenzae* d1 was achieved in subcutaneous tissue cages for up to 948 days. During persistence in the rabbits, similar changes in MOMP P2 of *H. influenzae* occurred, as observed in isolates from chronic bronchitis patients. In rabbits vaccinated with strain d3 and in nonvaccinated rabbits, antigenic drift occurred later than in rabbits vaccinated with strain d1. High titers of antibodies against *H. influenzae* were measured in tissue cage fluid and serum. Vaccination of the rabbits with *H. influenzae* d1 or d3, an antigenic variant of strain d1, resulted neither in eradication of *H. influenzae* d1 nor in increased antibody titers in serum and tissue cage fluid. The sera of nonvaccinated rabbits during persistence had no strain d1-specific bactericidal activity in the presence of complement. Vaccination with *H. influenzae* d1 induced serum bactericidal activity against strain d1 in the presence of complement. However, a variant of strain d1 appearing in the tissue cages was not killed by this serum bactericidal activity. We conclude that immunological pressure leads to the selection of MOMP variants of *H. influenzae* and that these variants escape the antibody-mediated strain-specific bactericidal activity against *H. influenzae*.

Nonencapsulated *Haemophilus influenzae* causing respiratory tract infections in patients with chronic bronchitis persists in the respiratory tract for months (9, 15, 19). Antibodies directed against a variety of antigens of the infecting *H. influenzae* strain are present in the sputum as well as in the serum (2, 7, 11, 15). During persistence, the major outer membrane proteins (MOMPs) P2 and P5 of *H. influenzae* change in electrophoretic mobility (8), resulting in antigenic drift in immunodominant epitopes (10). The changes in MOMP P2 are caused by accumulation of nonsynonymous point mutations, indicative of selective pressure (5). The in vitro selection of a MOMP P2 variant surviving the bactericidal activity of a MOMP P2-specific monoclonal antibody in the presence of complement (20) supports this assumption.

In this study, we investigated in a rabbit model whether immunological pressure induces the appearance of MOMP variants and whether variant-specific antibodies are elicited during persistence of nonencapsulated *H. influenzae* in subcutaneously implanted tissue cages. Similar models have been described for staphylococci in guinea pigs by Zimmerli et al. (28) and for gonococci in guinea pigs and rabbits by Veale et al. (23). The model allows repeated assessment of the number of persisting bacteria and analysis of their MOMPs and the immune response against these bacteria for months. We demonstrated that persistence and antigenic drift of *H. influenzae* occurred in the rabbit model similarly to those observed in patients with chronic bronchitis. The influence of intramuscular vaccination of rabbits with the same strain as inoculated in the tissue cages on antigenic drift and eradication of the bacteria was studied. In addition, the antibody response upon vaccination and the specificity of the serum bactericidal activity were determined. The pathophysiological significance of antigenic drift as an escape mechanism to circumvent the host defense during persistence of *H. influenzae* in the lower respiratory tract of patients with chronic bronchitis is discussed.

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MATERIALS AND METHODS

Bacteria and growth conditions. Nonencapsulated *H. influenzae* d1 and MOMP P2 variants d3 and d4 were isolated from sputum samples of patient T with chronic bronchitis (9). *H. influenzae* d1 was cultured overnight on chocolate agar plates and suspended in sterile phosphate-buffered saline (PBS; pH 7.4) for inoculation into the subcutaneous tissue cages in rabbits.

Animal model. New Zealand White rabbits (females) weighing 2.5 kg each were allowed free access to food and were housed individually throughout the experiments.

Tissue cages were prepared from silastic tubes (Dow Corning Corporation, Midland, Mich.) with an internal diameter of 6.35 mm, an external diameter of 9.50 mm, and a length of 125 mm. The tubes were perforated, resulting in regularly spaced holes (diameter, 2.5 mm), sealed at both ends, and heat sterilized prior to implantation.

In 19 rabbits, tissue cages were implanted. After the rabbits were anesthetized with 1 ml of Hypnorm, a 2-cm incision was made and the subcutaneous space was dissected bluntly. One tissue cage was implanted in each flank. The skin was closed with metal clips, which were removed after approximately 14 days.

Inoculation of tissue cages with *H. influenzae*. During 2 or 3 weeks after implantation, the subcutaneous tissue cages were allowed to become encapsulated by connective tissue and to be filled with sterile transudate as described previously (23, 25, 27). After this period, the tissue cages were inoculated. Overnight cultures of *H. influenzae* d1 from chocolate agar plates were suspended in sterile PBS (pH 7.4) in a concentration of 6×10^8 CFU/ml, and 1 ml of the suspension was injected with a 19-gauge needle through one of the perforations into each tissue cage.

The cages were sampled by withdrawing about 0.5 to 1.0 ml of fluid with a syringe and a needle through one of the holes in the cage. From 1 month on after inoculation, sampling was impossible unless 1 ml of sterile PBS was injected into the tissue cage before sampling. The number of bacteria persisting in the subcutaneous tissue cages was determined by colony counting of serial dilutions of tissue cage fluid in PBS on chocolate agar plates.

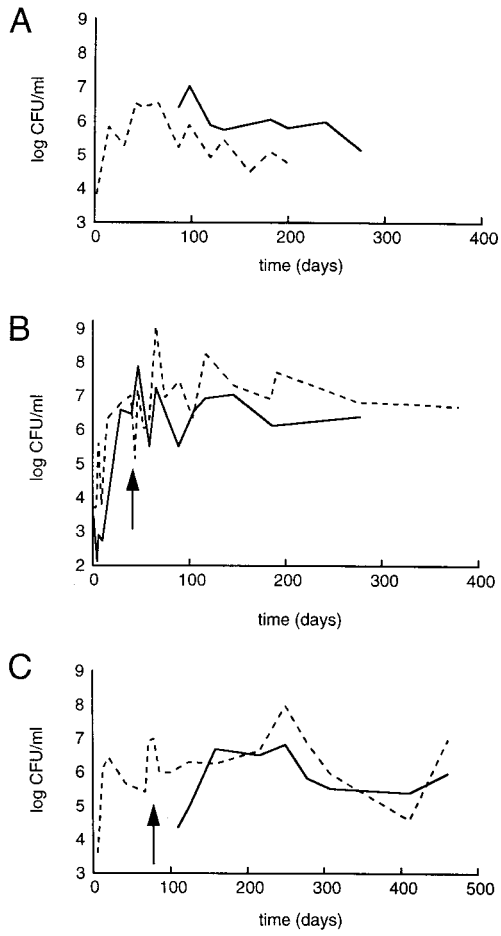

FIG. 1. Effect of vaccination on persistence of *H. influenzae* in tissue cages. The numbers of persisting *H. influenzae* d1 cells over time in the right (– – –) and left (——) tissue cages of a nonvaccinated rabbit from control group I (K158) (A), a rabbit from group II vaccinated with strain d1 on day 41 after inoculation (K199) (B), and a rabbit from group III vaccinated with strain d1 at day 68 after inoculation (K3032) (C) are shown. Note that in group III, the left tissue cage was inoculated after vaccination. The arrows indicate when the rabbits were vaccinated.

Vaccination protocol. The rabbits were stratified into four groups to study the relationship between antigenic drift of *H. influenzae* and immunologic pressure following vaccination. Six animals (control group) inoculated with *H. influenzae* d1 were not vaccinated (group I) to evaluate the antigenic drift in the absence of vaccination. Four rabbits were inoculated in both tissue cages with *H. influenzae* d1 and vaccinated with the homologous strain d1 6 to 8 weeks later (group II). Four rabbits were inoculated with *H. influenzae* d1 in one of the two tissue cages and vaccinated 6 to 10 weeks later with strain d1. After 5 to 14 weeks, the other cage was inoculated with strain d1 (group III) to investigate whether specific antibodies prevent the establishment of *H. influenzae* persistence in this latter tissue cage. Finally, both tissue cages of three rabbits were inoculated with *H. influenzae* d1, and after 6 to 10 weeks, these rabbits were vaccinated with the antigenic variant strain d3 (group IV).

The rabbits were vaccinated by intramuscular injection of formaldehyde-killed *H. influenzae* (2×10^8 CFU/ml). Formaldehyde killing was done by incubation of 2×10^8 CFU of *H. influenzae* per ml in 0.5% formaldehyde in PBS at 37°C for 16 h. The vaccination procedure took 3 weeks. On days 1, 3, and 5 of the first week, 0.25 ml of killed bacterial suspension was injected; on days 1 and 3 of the second week and on days 1, 3, and 5 of the third week, 1 ml of killed bacterial suspension was injected.

Screening for changes in MOMP P2 of *H. influenzae*. Each second week after inoculation, samples were collected from the tissue cages and 10 *H. influenzae* colonies cultured from each tissue cage were screened for MOMP changes. Screening for changes in electrophoretic mobility of the P2 and P5 proteins was performed by analyzing whole-cell lysates with sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Lugtenberg et al. (13), modified as described by van Alphen et al. (21).

Sequence analysis of the MOMP P2 gene. The MOMP P2 gene of the *H. influenzae* variants isolated from the subcutaneous tissue cages in rabbits was amplified by PCR using primers described previously (5). The obtained PCR fragments of the P2 gene were sequenced by using the *Taq* dye-primer cycle sequencing system with fluorescent dye-labeled −21M13 and M13 reverse primers as described before (5).

Samples for measurement of antibody titers and serum bactericidal activity. To determine serum antibody titers and serum bactericidal activity, blood samples were taken from the ear vein with a 21-gauge needle on the same day on which tissue cage fluid samples were taken for measuring antibody titers. Tissue cage fluid was centrifuged at 3,000 × *g* for 10 min to remove cellular debris, and the supernatant was used in the analysis.

Measurement of titers of antibodies directed against *H. influenzae*. Antibody (immunoglobulin G) titers directed against *H. influenzae* d1 or d3 were determined by whole-cell enzyme-linked immunosorbent assay (ELISA) (1) with protein A as a conjugate. A batch of human pooled serum from five healthy donors was taken as a standard to correct for variation in ELISA conditions. A titration curve was obtained by plotting the optical density as a function of the reciprocal serum dilution. The titer was defined as the dilution of serum or tissue cage fluid corresponding to an optical density of 0.2 above the background, as extrapolated from the slope of the curve.

Since the tissue cage fluid samples collected from 1 month on after inoculation were diluted by PBS, the antibody titers in tissue cage fluid were corrected relative to the concentration of albumin in tissue cage fluid and in serum, as determined by immunoturbidimetry (Cobas Bio Analyser) (17), using polyclonal goat anti-rabbit antialbumin (Pel-Freez Biologicals, Rogers, Ark.).

Serum bactericidal assay. The serum bactericidal activity was determined with a bactericidal assay described by van Alphen et al. (20). In short, bacteria grown to late exponential phase in brain heart infusion broth supplemented with NAD (10 mg/liter; Sigma N7381) and hemin (10 mg/liter; Sigma H2250) were suspended in 10 mM barbital buffer (pH 7.8) to a density of 4×10^5 CFU/ml. The incubation mixture consisted of 5 μl of this suspension; 5 μl of sodium barbital buffer containing 15 mM $CaCl_2$, 50 mM $MgCl_2$, and 3% (wt/vol) bovine serum albumin; 5 μl of serum from a patient with agammaglobulinemia as a complement source; and 15 μl of adsorbed, heat-inactivated (30 min, 56°C) rabbit serum. In experiments in which the bactericidal activity against an antigenic variant was tested, 10% fetal calf serum was added to the incubation mixture since the variants in a control incubation mixture without serum exerted killing. Sodium barbital buffer was added to a final volume of 50 μl. After 30 and 60 min of incubation, 10-μl samples were taken and plated on chocolate agar plates for counting the number of surviving CFU.

Serum was adsorbed with a heterologous *H. influenzae* strain, d4, by the method of van Alphen et al. (22) to remove serum components with bactericidal activity present in all rabbit serum samples, even in the absence of detectable antibodies specific for *H. influenzae* d1. Adsorption did not result in a significant reduction in the antibody titer. *H. influenzae* d4 grown in brain heart infusion broth supplemented with NAD and hemin was harvested by centrifugation. The packed cells were suspended in an equal volume of serum. The suspension was incubated at 37°C for 30 min and then at 4°C for 16 h. The adsorbed serum was recovered by centrifugation and stored at −70°C. Adsorption with *H. influenzae* d1 or a nonrelated strain was performed by using the same procedure.

Statistics. Statistical analysis of the appearance of the first variant in three groups of rabbits was performed with the Wilcoxon rank sum test. *P* values of <0.05 were considered to be significant. Data on the bactericidal activity of rabbit serum were analyzed with Student's *t* test.

Nucleotide sequence accession numbers. The nucleotide sequence data for variants c1, c2, e1, and e2 appear in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession numbers X92382, X92383, X92384, and X92385.

RESULTS

Persistence of *H. influenzae* in the subcutaneous tissue cages in rabbits. Nonencapsulated *H. influenzae* inoculated in the 38 tissue cages 2 or 3 weeks after implantation of the tissue cages survived in 17 of the 19 rabbits. After inoculation of the tissue cages, the number of bacteria per milliliter of tissue cage fluid increased rapidly and then persisted at a constant level of approximately 6×10^6/ml. The number of persisting bacteria per milliliter over time is shown in Fig. 1A for one rabbit representative of the nonvaccinated rabbits (group I). Tissue and lavage fluid from the compartment outside the tissue cage remained culture negative, indicating that the bacteria did not spread or survive outside the tissue cage. Persistence of *H. influenzae* in the tissue cages proceeded as long as the tissue cages remained subcutaneously or the rabbits survived (up to 948 days).

In rabbits inoculated with *H. influenzae* d1 and vaccinated

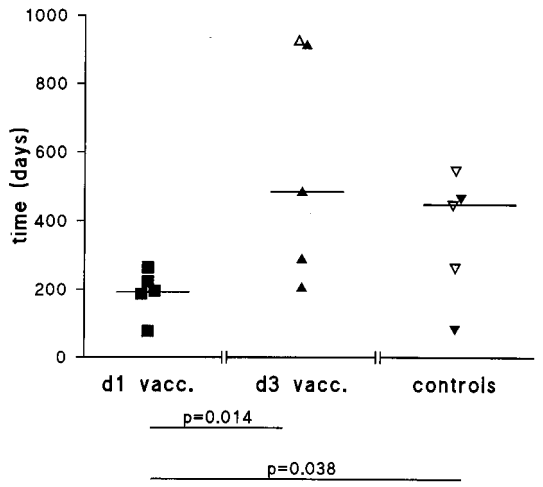

FIG. 2. Appearance of the first antigenic variant of *H. influenzae* d1 (closed symbols) over time in rabbits vaccinated with *H. influenzae* d1 (d1 vacc., group II; ■) and strain d3 (d3 vacc., group IV; ▲) and in nonvaccinated rabbits (controls, group I; ▼). The day of the inoculation was taken as day zero. The tissue cages lost before an MOMP P2 variant of *H. influenzae* d1 was cultured from that tissue cage are represented by open symbols. The median value is indicated for each group.

with strain d1 (group II) or vaccinated with the variant strain d3 (group IV), the number of bacteria per milliliter of tissue cage fluid remained similar to the number of bacteria in the control rabbits (group I) (Fig. 1B). Even *H. influenzae* d1 inoculated in one of the two tissue cages in one rabbit after vaccination of the rabbit with strain d1 (group III) multiplied and persisted in the tissue cages (Fig. 1C).

Appearance of antigenic variants in nonvaccinated rabbits. Screening of the rabbits over time to assess the appearance of the first MOMP variant revealed that the first MOMP variant in nonvaccinated rabbits (group I) was detected 84 days after inoculation. Substantial variation in the time of appearance of the first MOMP variant was observed among individual rabbits (Fig. 2) and between two tissue cages in individual rabbits. Therefore, appearance of the first MOMP variant over time was analyzed for each tissue cage separately.

The first MOMP P2 variants appeared later in the group inoculated with strain d1 and vaccinated with the heterologous strain d3 (group IV; median, day 482; $P = 0.014$) and in the nonvaccinated rabbits inoculated with strain d1 (group I; median, day 457; $P = 0.038$) than in the group of rabbits inoculated and vaccinated with strain d1 (group II; median, day 195) (Fig. 2). Some nonvaccinated rabbits lost a tissue cage before antigenic variants were cultured from their tissue cages. When a rabbit lost a tissue cage later than 264 days after inoculation, the last day that a variant appeared in the rabbits vaccinated with *H. influenzae* d1, the time points of extrusion of these tissue cages were included in the calculation, since the statistical analysis of the data would not be influenced if a MOMP variant were to appear later on. If the tissue cage was lost earlier than 264 days after inoculation, the result from that tissue cage was excluded from the analysis. Because of insufficient data from group III, the tissue cages from this group were also excluded from the analysis.

MOMP P2 variants persisted together with the original strain (d1) in 10 tissue cages (2 in group I, 3 in group II, 2 in group III, and 3 in group IV), but in 5 tissue cages, the original strain was outgrown by the variant (1 in group I, 2 in group II, 1 in group III, and 1 in group IV). In five tissue cages, a second MOMP P2 variant appeared after appearance of the first variant. In one tissue cage from rabbit K159, even a third variant was identified.

Characteristics of MOMP P2 variants isolated from the tissue cages. The variation in MOMP P2 of variants of *H. influenzae*, differing in the molecular weight of MOMP P2 from strain d1, obtained from tissue cages, was identified with sequence analysis. Alignment of the deduced amino acid sequences of MOMP P2 from variants b1 to b4 isolated from a nonvaccinated rabbit has been published elsewhere (5). The amino acid sequences of loops 5 and 6 of variants c1 and c2 obtained from a rabbit vaccinated with strain d1 (group I) and of variants e1 and e2 from a rabbit vaccinated with strain d3 (group IV) are shown in Fig. 3. Also included are data for variants d1 to d4 obtained from a patient with chronic bronchitis. All antigenic variants contained DNA point mutations that resulted in amino acid substitutions (nonsynonymous mutations) in loop 6. MOMP P2 variants obtained after longer persistence of variant d1 (b4 and c2) also showed an amino acid substitution in loop 5 as a consequence of nonsynonymous nucleotide substitutions. In the other loops of MOMP P2, no variation was detected. Comparable types of amino acid changes were observed in all variants, or the same types of nucleotide substitutions were involved.

Antibodies in serum and tissue cage fluid directed against *H. influenzae*. The appearance of antibodies in sera and tissue cage fluid of the rabbits was determined with a whole-cell ELISA with *H. influenzae* d1 as the antigen. In rabbits inoculated only with *H. influenzae* d1 (group I), the serum antibody titers against *H. influenzae* d1 increased rapidly after inoculation and then remained at a constant level of approximately $1/10^5$ (Fig. 4a).

The development of serum antibody titers to *H. influenzae* d1 over time is shown for three representative rabbits vaccinated either with strain d1 (group II) or strain d3 (group IV) in Fig. 4B and C, respectively. The serum antibody titers of all rabbits after (0 to 15 days) inoculation, prior to (1 to 18 days) vaccination, and 43 to 64 days postvaccination are summarized in Table 1. After vaccination of the rabbits with either the homologous strain d1 (group II) or its variant d3 (group IV), the serum antibody titers did not differ from those in nonvaccinated rabbits (group I).

Since the persisting bacteria are in contact with the antibodies in the tissue cages, the antibody titers in tissue cage fluid were compared with the antibody titers in serum. One month after inoculation of the tissue cages, undiluted tissue cage fluid was obtained from the cages, but later the tissue cage fluid had to be diluted before a sample could be taken because of high viscosity of the tissue cage fluid. Therefore, the antibody titers

Rabbit	Variant	Loop 5	Loop 6
	d1	YKTAGADFDPYGDFGLGRK	TKYYTTT-DSSSDSQTITNPAYDEKRSF
	d2	FS	H T DDGR
	d3	FS	H T GDGR
	d4	FS	H T GGGR
183	b1		G
	b2		N GN K
	b3		N GN K
	b4	S	A GN K
219	c1		N
	c2	N	G N
159	e1		G
	e2		GG T

FIG. 3. Deduced amino acid sequences of MOMP P2 loops 5 and 6 from *H. influenzae* variants d1 to d4, isolated from a patient with chronic bronchitis; variants b1 to b4, isolated from a nonvaccinated rabbit (K183, group I); variants c1 and c2, isolated from a rabbit vaccinated with strain d1 (K219, group II); and variants e1 and e2, isolated from a rabbit vaccinated with strain d3 (K159, group IV). The sequences of d1 to d4 and b1 to b4 are included for reference (6).

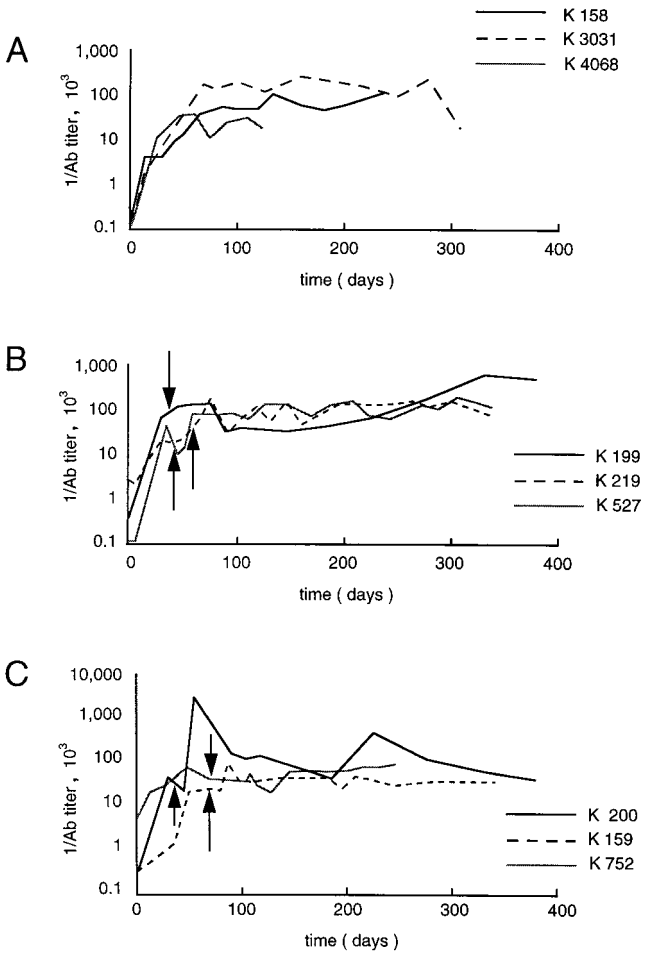

FIG. 4. Titers in serum of antibody (Ab; immunoglobulin G) against *H. influenzae* d1 during persistence of *H. influenzae* in subcutaneous tissue cages in nonvaccinated rabbits (group I) (A) and rabbits vaccinated with *H. influenzae* d1 (group II) (B) and d3 (group IV) (C). The arrows indicate when the rabbits were vaccinated.

were corrected for the dilution of the tissue cage fluid. Since the presence of albumin in tissue cage fluid was assumed to be the result of passive diffusion, albumin was chosen as the reference protein. The ratio of the concentration of albumin of undiluted tissue cage fluid to serum was 0.61 ± 0.20. This ratio was taken as a constant factor to correct for the dilution of the tissue cage fluid, since any variation up to a factor of 4 would hardly affect the antibody titers, which are presented in a log scale (Fig. 5).

The antibody titers against *H. influenzae* d1 increased rapidly in both tissue cages after inoculation, as was observed for serum antibodies, and remained high at a constant level during *H. influenzae* persistence in the tissue cages (Fig. 5A). Vaccination with strain d1 did not result in higher antibody titers in the tissue cages and serum samples than in nonvaccinated rabbits (Fig. 5B).

To investigate the cross-reactivity of the antibodies directed against *H. influenzae* d1, ELISAs with strain d3 as the antigen were performed. The antibody titers of sera and tissue cage fluid of d3-vaccinated rabbits (group IV) and of d1-vaccinated rabbits (group II) were compared. The serum antibody titers specific for bacteria of strains d1 and d3 in a representative rabbit from group II and a representative rabbit from group IV are shown in Fig. 6. The titers of the antibodies directed against strain d3 did not differ significantly in the sera of these two groups of rabbits, since the ranges of the antibody titers of the two groups of rabbits overlapped. The serum antibody titers against strains d3 and d1 were similar in the two groups of rabbits. These results indicate that the majority of antibodies in serum were directed against *H. influenzae* antigenic determinants present on strain d1 as well as on strain d3.

Serum bactericidal activity. It has been shown previously that in the presence of complement, the bactericidal antibodies directed against *H. influenzae* are strain specific (6, 16, 20). We investigated whether antigenic drift occurs in response to strain-specific bactericidal antibodies and whether the antigenic variants appearing in the rabbit are resistant to the bactericidal activity of antibodies elicited before the variants appeared. Since the titers (Fig. 5) and specificity, as determined by immunoblotting (data not shown), of the antibodies in tissue cage fluid and serum were similar and the tissue cage fluid was viscous, contained tissue debris, and was diluted by PBS, the experiments were performed only with serum samples as antibody sources.

Sera from rabbits prior to inoculation with *H. influenzae* (0-serum) were bactericidal for *H. influenzae*, although antibodies to *H. influenzae* could not be detected with ELISA. After adsorption of 0-serum with the heterologous strain d4 or a nonrelated *H. influenzae* strain, the bactericidal activity of the 0-serum against *H. influenzae* d1 was abrogated (survival of 82% ± 12% or 126% ± 57% [mean ± standard deviation], respectively). Adsorption with either *H. influenzae* d4 or a nonrelated *H. influenzae* strain did not reduce the bactericidal activity of postvaccination serum (see below). Although cross-reactive antibodies may have been removed by adsorption, strain-specific antibodies were still present (16).

H. influenzae d1 and the variants appearing in the rabbits were not killed by complement in the absence of antibodies. The results of the bactericidal assays are summarized in Fig. 7. In two (K3031 and K754) of three nonvaccinated rabbits (group I), specific serum bactericidal activity against *H. influenzae* d1 was hardly observed in various samples taken during persistence of *H. influenzae*, since at least 52% of the bacteria survived the assay conditions. In one rabbit (K158), the number of bacteria decreased below 25% in the presence of serum taken at day 119 or later after inoculation, indicating significant killing (Fig. 7A).

The effect of vaccination with *H. influenzae* d1 on the appearance of bactericidal activity of the serum for the inoculated strain is shown in Fig. 7B. In the presence of complement and serum from two rabbits (K199 and K219) vaccinated with strain d1 (group II), the number of surviving bacteria decreased below 25% just after vaccination. In one rabbit (K527) vaccinated with strain d1, the serum was bactericidal for strain d1 before vaccination.

To analyze whether antigenic variants appeared in response to serum bactericidal activity directed against *H. influenzae* d1, the serum bactericidal activities against strain d1 and the first antigenic variant appearing in a rabbit were compared. Figure 7C shows that exposure of the variant to serum of nonvaccinated rabbit K754 did not result in complement-dependent killing of that variant appearing in the rabbit. Serum samples collected before appearance of an antigenic variant from two d1-vaccinated rabbits (K199 and K219, group II) with complement-dependent bactericidal activity against strain d1 had no bactericidal activity against the variants appearing later in the tissue cages. Only serum samples of rabbit 527 taken at day 84 and day 244 after inoculation killed the variant that appeared later (day 223 after inoculation) (Fig. 7D). The bactericidal

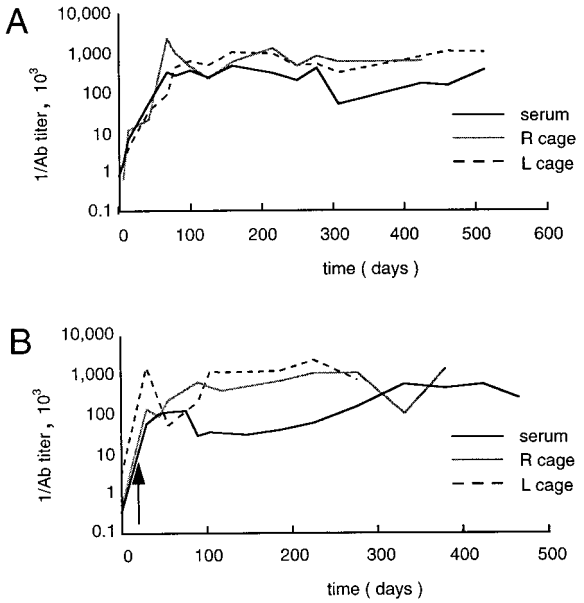

FIG. 5. Titers of antibodies (Ab) directed against *H. influenzae* d1 in tissue cage fluid from the right (R) and left (L) tissue cages and in sera of a nonvaccinated rabbit (K3031, group I) (A) and a rabbit vaccinated with *H. influenzae* d1 (K199, group II) (B). The arrow indicates when the rabbit was vaccinated.

activity of this serum was abrogated after adsorption with *H. influenzae* d1 (survival, 93% ± 5% [mean ± standard deviation]).

DISCUSSION

H. influenzae persisted in the subcutaneous tissue cages in rabbits. Antigenic drift of *H. influenzae*, occurring during persistence, was the consequence of nonsynonymous point mutations in the MOMP P2 gene. We showed that antigenic variants appeared later in nonvaccinated rabbits and rabbits vaccinated with the heterologous strain than in rabbits vaccinated with the homologous strain. The specific serum bactericidal activity after vaccination with the homologous *H. influenzae* strain was directed against the inoculated strain but not against the antigenic variant.

Zimmerli et al. (28), who described a similar animal model in guinea pigs, reported that 4 weeks after implantation of tissue cages in guinea pigs, rich vascularized tissue containing lymphocytes, fibroblasts, and collagen fibers was in close contact with the tissue cages. In this foreign body-induced inflammatory reaction, the phagocytic and bactericidal capacity of the polymorphonuclear leukocytes has been shown to be impaired (27, 28). The polymorphonuclear leukocytes were thought to be damaged by a temporary contact with the foreign body to which they had been attracted, thereby exhausting their functional capacity (12, 27, 28). Another determinant of the foreign body infection is the formation of a bacterial glycocalyx which reduces the bacterial accessibility to phagocytes (4). In two rabbits, we could not establish a persistent infection with *H. influenzae* in the tissue cages in the rabbits. This phenomenon was also reported for *Neisseria gonorrhoeae* by Veale et al. (23), who observed that persistent infections occurred only when the initial infiltration of polymorphonuclear leukocytes on the first days was low.

Persistence of *H. influenzae* in tissue cages in rabbits resembled persistence in patients with chronic bronchitis, since antigenic variants which had changes in the MOMP P2 composition similar to those observed in the chronic bronchitis isolates appeared during persistence (Fig. 3). A distinct difference between the antigenic variants in patients and the rabbit model is that MOMP P5 alterations were not found among variants isolated from the tissue cages in rabbits. These results indicate that antigenic drift is probably just the consequence of persistence of *H. influenzae* in a particular host environment. In patients with chronic bronchitis, the local conditions may obviously favor persistence as well as selection of antigenic variants. Apparently, the presence of an implanted tissue cage also creates conditions required for persistence and antigenic drift of *H. influenzae*, although the selective conditions for MOMP P5 variants may be different.

TABLE 1. Serum antibody titers to *H. influenzae* d1 in rabbits

Rabbit[a]	1/titer of antibodies (10^4)[b]		
	Postinoculation[c]	Prevaccination[d]	Postvaccination[c]
Nonvaccinated (group I)			
K158	0.018		4.6
K3031	0.028		18.0
K754	0.440		5.1
K4066	0.180		6.1
K4067	0.013		3.4
K4068	0.011		3.0
d1 vaccinated (group II)			
K199	0.034	6.1	3.7
K219	0.193	2.0	6.0
K527	<0.002	4.1	7.9
K4069	0.086	3.4	16.4
d1 vaccinated (group III)			
K162	0.041	2.0	6.7
K3032	0.013	1.3	31.0
K753	1.200	19.0	19.0
K530	0.026	2.0	8.7
d3 vaccinated (group IV)			
K200	0.015	1.1	6.7
K159	0.057	2.2	3.8
K752	0.500	4.2	6.2

[a] All were inoculated with *H. influenzae* d1.
[b] Determined by whole-cell ELISA; preinoculation titers were below 0.050.
[c] Zero to 15 days after inoculation.
[d] One to 18 days before vaccination (i.e., 30 to 68 days after inoculation).
[e] Forty-three to 64 days after vaccination (i.e., 97 to 125 days after inoculation); 90 to 109 days after inoculation in nonvaccinated rabbits.

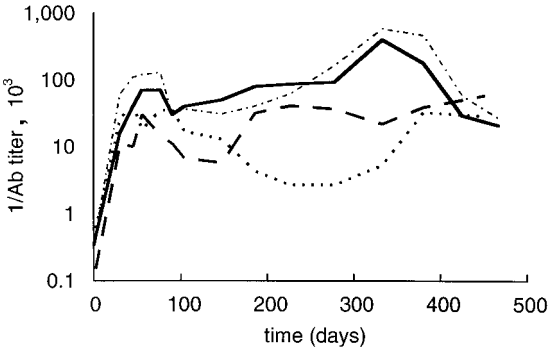

FIG. 6. Cross-reactivities of serum immunoglobulin G antibodies (Ab) specific for *H. influenzae* d1 cross-reacting with strain d3 in a rabbit vaccinated with strain d1 (K199, group II; · – · –, titer of antibodies directed against strain d1; ——, titer of antibodies directed against strain d3) and a rabbit vaccinated with strain d3 (K200, group IV; — —, titer of antibodies directed against strain d1; · · ·, titer of antibodies directed against strain d3), both vaccinated 41 days after inoculation.

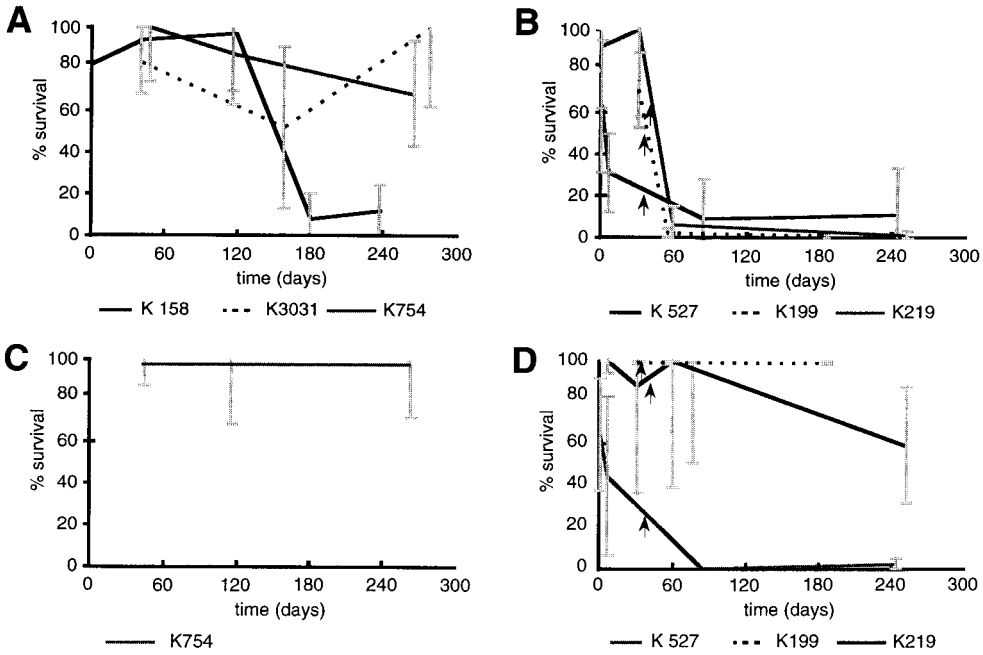

FIG. 7. (A and B) Survival of *H. influenzae* d1 in the bactericidal assay with sera from three nonvaccinated rabbits (group I) (A) and from three rabbits vaccinated with *H. influenzae* d1 (group II) (B). (C and D) Survival of the first MOMP P2 variant of *H. influenzae* d1 appearing in a rabbit in the bactericidal assay in the presence of sera from a nonvaccinated rabbit (group I) (C) and from rabbits vaccinated with *H. influenzae* d1 (group II) (D). The arrows indicate when the rabbits were vaccinated.

H. influenzae persisted in the subcutaneous tissue cages despite the presence of high titers of antibodies in serum and tissue cage fluid during persistence. High titers of antibodies directed against a large variety of *H. influenzae* antigens were also found in the serum and sputum samples of patients with chronic bronchitis (7).

Selective pressure of MOMP P2 variants was indicated by the fact that only nonsynonymous mutations occurred and by the significantly later appearance of MOMP variants in control rabbits and in rabbits vaccinated with the heterologous strain than in rabbits vaccinated with the homologous strain. The original strain and the MOMP variant coexisted in the tissue cage, indicating that the selective pressure is not very strong. The selective pressure may be mediated by the antibody-mediated effector mechanisms complement-dependent killing and opsonophagocytosis (6, 18, 20), both requiring complement activation. Whereas after vaccination the titers of the antibodies directed against the bacteria did not increase, serum complement-dependent bactericidal activity against the inoculated strain was detected in the rabbits just after vaccination with the homologous *H. influenzae* strain. This bactericidal activity is probably also present in the tissue cage fluid after vaccination, since the titers and specificities of the antibodies were similar to those in serum as a result of diffusion of serum proteins into the tissue cages. This discrepancy between the results of ELISAs and bactericidal assays was previously described by Groeneveld et al. (10). Selection can also be mediated by phagocytosis (18), but *H. influenzae* is poorly opsonophagocytized (24), and opsonophagocytosis is more inefficient in foreign body-associated infections (27, 28).

Wallace et al. showed that in an animal model, clearance of *H. influenzae* was accelerated and appeared to be independent of the amount of local or systemic antibodies, but this clearance was felt to be mediated by sensitized T lymphocytes (26). We cannot exclude that immunological mechanisms, like T-cell-mediated immunity, play a role in the process of antigenic drift. However, the findings that MOMP P2 variants appear later in d3-vaccinated rabbits (group IV) than in d1-vaccinated rabbits (group II) and that the serum bactericidal activity was specific for the inoculated *H. influenzae* d1 indicate strongly that the selective pressure was dependent on the development of antibodies. Vaccination may influence the properties of antibodies, thereby increasing the immunological pressure, as demonstrated in studies with *Pseudomonas aeruginosa* in which vaccine-induced antibodies exhibited affinities that were at least 100-fold higher than those of naturally acquired antibodies (3).

We conclude that MOMP P2 variants of *H. influenzae* persisting in subcutaneous tissue cages in rabbits are selected under pressure of the variant-specific serum bactericidal activity elicited by vaccination with the homologous strain. The selection process is slow, probably because of inefficiency of antibody-mediated defense mechanisms, especially opsonophagocytosis (24). Often the antigenic variant and the original strain both persisted in the tissue cages for some time. This phenomenon has been observed frequently in patients with cystic fibrosis but is not obvious in patients with chronic bronchitis (14). The growth advantage of the antigenic variants under the selective conditions is limited compared with the growth of the original strain. Especially during exacerbations, the selective mechanisms in patients with chronic bronchitis seem to be more efficient than those in patients with cystic fibrosis and in the subcutaneous tissue cages in rabbits. Lack of antibody-mediated bactericidal activity against the inoculated *H. influenzae* strain and poor opsonophagocytosis of nonencapsulated *H. influenzae* previously reported (24) offer explanations for the inefficiency of the immunological selective pressure in nonvaccinated rabbits and patients with chronic bronchitis. It will be difficult to prove that similar mechanisms of selective pressure are operative in patients with chronic

bronchitis, since the time interval between infection and appearance of variants cannot be determined.

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